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Embryotoxicity Screening Using Embryonic Stem Cells in vitro: Correlation to in vivo Teratogenicity

Gabriele Scholz^a Ingeborg Pohl^a Elke Genschow^b Martina Klemm^a
Horst Spielmann^a

^aCenter for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the

^bFederal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Berlin, Germany

Key Words

Differentiation in vitro · Embryonic stem cell test · Cardiomyocytes · Cytotoxicity · Embryotoxicity · Prediction model

Abstract

Blastocyst-derived pluripotent embryonic stem (ES) cells of the mouse can be induced to differentiate in culture into a variety of cell types, including cardiac muscle cells. The embryonic stem cell test that makes use of the differentiation of ES cells into cardiomyocytes in a standardized in vitro model was developed to offer an alternative method to comprehensive in vivo studies in reproductive toxicology about toxic effects of chemicals. ES cells of the mouse cell line D3 are investigated for their preserved capability to differentiate following drug exposure, and both ES cells and differentiated fibroblast cells of the mouse cell line 3T3 are comparatively analyzed for effects on viability. The following endpoints are used to classify the embryotoxic potential of chemicals into three classes of in vitro embryotoxicity (non-, weakly or strongly embryotoxic). These endpoints are: (1) the inhibition of differentiation of ES cells into cardiomyocytes after 10 days of treatment, and the decrease of viability (cytotoxicity) of (2) 3T3 cells and (3) ES cells after 10 days

of treatment, determined by a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) test. 50% inhibition concentrations for differentiation (ID_{50}) and cytotoxicity ($IC_{50}D3$ and $IC_{50}3T3$) are calculated from concentration-response curves. Applying linear analysis of discriminance, a biostatistical prediction model (PM) was developed. This procedure identified three variables, the $Ig(IC_{50}D3)$, the $Ig(IC_{50}3T3)$ and the relative distance b between $IC_{50}3T3$ and ID_{50} , that improved the separation of the three classes of embryotoxicity compared to the pr -

Abbreviations used in this paper

Asc	ascorbic acid
DPH	diphenylhydantoin
EB	embryoid body
ES cells	embryonic stem cells
EST	embryonic stem cell test
5-FU	5-fluorouracil
LIF	leukemia inhibitory factor
MM	micromass
MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
PM	prediction model
RA	retinoic acid
WEC	whole embryo culture

diction mod I that was originally proposed after test development. Unlike the original PM, the improved PM incorporates as one variable the relative distance between IC_{50} 3T3 and ID_{50} , instead of the ratio ID_{50}/IC_{50} D3 that was used previously.

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Introduction

Developing organisms undergo rapid and complex changes within a relatively short period of time. Therefore, in mammals any agent administered during pregnancy to the mother under appropriate conditions of time and dosage may interfere with embryonic development and induce embryo lethality, growth retardation or teratogenic effects, with structural and functional abnormalities in the offspring. Persistent lesions, e.g. general growth retardation or delayed organ growth, are defined as embryotoxic effects. Currently, according to the great number of existing industrial chemicals that are commercially available, screening tests or multigeneration studies must be conducted to provide information on the toxic effects on specific elements of the highly complicated reproductive cycle according to guidelines of the OECD [OECD Test Guidelines No. 414, 1981; No. 415, 1983; No. 416, 1983; No. 421, 1995; No. 422, 1996]. For chemicals used as drugs, segment studies have to be conducted covering preconceptual exposure as well as pre- and postnatal development including the lactation period [guidelines of the International Conference on Harmonisation, ICH, 1993].

These in vivo protocols are time-consuming, expensive and they are carried out on large numbers of laboratory animals. One important feature of the in vivo test protocols is the assessment of maternal toxicity in comparison to adverse effects in the offspring. Today, these two important aspects can to some extent be covered by in vitro approaches. In developmental toxicology, many in vitro alternatives to testing in animals have been developed using a wide spectrum of cell and tissue cultures, e.g. permanent cell lines, cultures of primary embryonic cells and cultures of nonmammalian tissue and mammalian embryos [for a review, see Brown et al., 1995; Spielmann, 1998]. For example, in the rat limb bud micromass (MM) assay, effects on the viability of primary limb bud cell cultures (cytotoxicity) are compared to effects on the differentiation of these cells into chondrocytes [Flint and Orton, 1984]. In the postimplantation rat whole embryo culture (WEC) assay, both general growth retardation and

specific malformations of the cultivated embryos are assessed [for a review, see Spielmann, 1998].

One of the more recently developed in vitro approaches is based on blastocyst-derived pluripotent embryonic stem (ES) cells of the mouse. In contrast to the WEC and MM tests, the embryonic stem cell test (EST) [Spielmann et al., 1997] has the advantage of using established cell lines without the need to sacrifice pregnant animals.

Since cultivation and maintenance of ES cells in the undifferentiated state was first reported by Evans and Kaufman [1981], cultivation and differentiation of ES cells is today a widely applied method in mammalian developmental biology. ES cells can be genetically manipulated to generate transgenic or 'knockout' mice [Thomas and Capecchi, 1987], and in vitro cell culture models were established to study myogenesis, angiogenesis, hematopoiesis, neurogenesis and cardiogenesis in the mouse [Wiles and Keller, 1991; Wobus et al., 1991; Heuer et al., 1994; Maltsev et al., 1994; Rohwedel et al., 1994; Strübing et al., 1995; Kolossov et al., 1998].

The differentiation of ES cells into cardiac cells [Doetschman et al., 1985] has been used in investigations on prenatal pharmacology, electrophysiology and molecular genetics [Wobus et al., 1991; Maltsev et al., 1993; Metzger et al., 1996; Kolossov et al., 1998]. The EST developed by Spielmann and coworkers [1997] was designed to adopt the ES cell cultures in embryotoxicity studies by using an endpoint related to prenatal differentiation *in vivo*, the differentiation of ES cells into contracting cardiac muscle cells. The inhibition of this specific differentiation by embryotoxic agents was compared to cytotoxic effects in the ES cells [Laschinski et al., 1991]. To improve the predictive potential of this in vitro embryotoxicity test, in the EST, the influence of potentially embryotoxic chemicals on the viability of ES cells and on their capability to differentiate *in vitro* [Wobus et al., 1991; Rohwedel et al., 1994; Spielmann et al., 1997] is examined in the permanent mouse ES cell line D3 and compared to the effect on viability of differentiated fibroblast cells of the line 3T3.

Despite the use of some established alternative embryotoxicity tests like the MM test and the WEC test in the drug and chemical industry for screening new chemicals that are structurally related to established embryotoxic chemicals, none of these in vitro tests has to date been sufficiently validated for regulatory purposes according to the recommendations of the European Centre for the Validation of Alternative Methods (ECVAM) [Balls et al., 1995]. Therefore, in developmental toxicity

testing there is a strong demand for validated in vitro tests using mammalian embryos as well as primary cultures of embryonic cells and permanent cell lines. The limiting factors for in vitro screening are obvious when taking into account the complexity of normal differentiation and development of mammalian embryos. Nevertheless, three of the most promising in vitro tests are currently undergoing formal validation in a European ring trial: the WEC test, the MM test and the EST. This validation study is aimed at predicting the embryotoxic potential of a set of test chemicals characterized by high-quality in vivo embryotoxicity data in laboratory animals and humans [Scholz et al., 1998; Genschow et al., 1999].

In this study the performance in the EST to predict the embryotoxic potential of test chemicals is described and we report on the development of an improved prediction model for the EST, derived from results obtained in our laboratory in a prevalidation trial that preceded the currently running validation study. The importance of sound biometrical prediction models as a means for an objective evaluation and classification of the performance of in vitro tests in validation studies is discussed.

Test Procedure of the EST

BALB/c 3T3 cells (clone 31, ICN Flow, Eschwege, Germany) are routinely maintained in DMEM supplemented with 10% FCS, 4 mM glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin. ES cells (line D3, a donation from Prof. R. Kemler, MPI, Freiburg, Germany) are maintained in DMEM supplemented with 20% FCS, 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 1% nonessential amino acids, 0.1% β -mercaptoethanol and 1,000 U/ml leukemia inhibitory factor (LIF) [Williams et al., 1988].

To determine cytotoxic effects on ES and 3T3 cells, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) tests are performed in the absence of LIF as described previously [Spielmann et al., 1997]. Briefly, 500 cells are seeded into each well of a 96-well microtiter plate and grown in the presence of a concentration range of the test chemical. A negative control containing solvent diluted in medium is also included. After 10 days of culture with two changes of medium (containing the appropriate concentration of test chemical) on days 3 and 5, the viability of the cells is determined using an MTT test, which is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow substrate MTT into a dark blue formazan product, which is de-

tected quantitatively using a microplate ELISA reader. Cytotoxicity is expressed as the concentration decreasing viability to 50% of the control level ($IC_{50}3T3$ and $IC_{50}D3$; determined from a concentration-response curve).

To detect effects on the differentiation of ES cells into cardiomyocytes, a differentiation assay is performed as described previously [Spielmann et al., 1997]. Briefly, 750 cells in drops of 20 μ l are seeded (in the absence of LIF to allow differentiation) into the lid of a culture dish and grown for 3 days in the 'hanging drop' culture in the presence of a concentration range of the test chemical. During this period the cells will form aggregates called embryoid bodies (EBs). After 3 days of 'hanging drop' culture the EBs are transferred to bacterial petri dishes containing the appropriate concentration of the test chemical for another 2 days. Bacterial petri dishes are used to avoid adherence and outgrowth of the EBs during this stage of the culture. On day 5 EBs are seeded separately into wells of a 24-well tissue culture plate (containing the appropriate concentration of test chemical) to allow adherence and outgrowth of the EBs and development of contracting cardiomyocytes. Differentiation is assessed by microscopic inspection of the EBs on day 10. The percentage of wells of each plate in which contracting cardiomyocytes have developed is determined and compared to the percentage of wells in which cardiomyocytes developed on the solvent control plate. The inhibition of differentiation (ID_{50}) is expressed as the concentration of the test chemical inhibiting the development into contracting cardiomyocytes by 50% (calculated from the concentration-response curve).

Classification of Embryotoxic Chemicals

In a recent prevalidation study that was conducted according to the recommendations of the European Centre for the Validation of Alternative Methods (ECVAM) [Curren et al., 1995; Soutice and Curren, 1997], chemicals assigned to the three classes of embryotoxicity were tested under blind conditions in order to prove that the assay is reproducible and transferable to other laboratories and that it is able to predict the embryotoxic potential of a given set of chemicals.

In embryotoxicity testing the selection of test chemicals is particularly difficult, since the number of chemicals which are backed by high-quality in vivo data is rather limited. For the prevalidation trial, only test chemicals were used for which in vivo data of a sufficiently high quality were available from both testing in animals and human pregnancy. These were selected from a list of

Table 1. Set of test chemicals

Embryotoxicity in vivo	Test chemical	CAS No.	In vivo activity species	Use
Class 1: nonembryotoxic	Penicillin G	69-57-8	—	Antibiotic
	Isoniacide	54-85-3	—	Tuberculostatic
	Ascorbic acid	134-03-2	—	Vitamin
Class 2: weakly embryotoxic	Diphenylhydantoin	630-93-3	Human, animal	Anticonvulsant
	Caffeine	58-08-2	Human, animal	Central stimulant, drug
	Dexamethasone	50-02-2	Human, animal	antiasthmatic, glucocorticoid
Class 3: strongly embryotoxic	Cytosine arabinoside	69-74-9	Human, animal	Cytostatic
	All-trans-RA	302-79-4	Human, animal	Acne therapy
	Hydroxyurea	127-07-1	Animal	Cytostatic
	5-Fluorouracil	51-21-8	Animal	Cytostatic

Characteristics of the test chemicals used in the study are described: 9 test chemicals and the positive control 5-FU. The embryotoxicity in vivo and their use in humans are given. Test chemicals with known embryotoxic potential in vivo were selected from a published list recommended by the US Teratology Society for the validation of in vitro embryotoxicity tests [Smith et al., 1983]. All chemicals were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Chemicals were dissolved according to a strategy developed for dissolving of coded chemicals, which has been included in the standard protocol. Briefly, a hierarchical approach was used to find an appropriate concentration in a suitable solvent, starting with PBS or medium as solvent, then 50% (vol/vol) ethanol in PBS, then DMSO, and finally pure ethanol.

chemicals recommended by the US Teratology Society for in vitro teratogenesis test validation [Smith et al., 1983]. Nine test chemicals and a positive control of known embryotoxic potential in vivo were selected for testing during the prevalidation study (table 1), mainly consisting of drugs which have been used in human pregnancy, e.g. in case of bacterial infections or the therapy of cancer. The prevalidation trial was conducted in two European and one US laboratory, yet the exact results obtained during this interlaboratory study are reported elsewhere [Scholz et al., 1999]. This report focuses exclusively on the results of our laboratory, since they were used as the basis for the development of an improved PM.

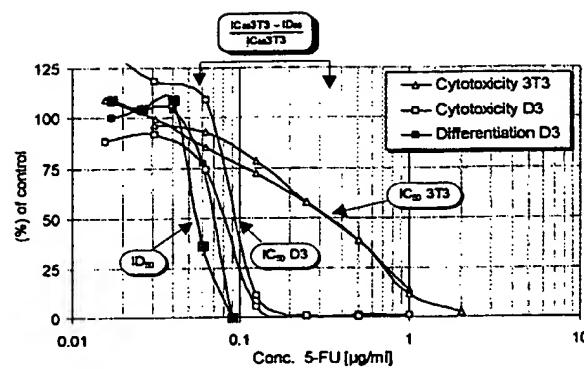
Most of the chemicals tested (table 1) can be categorized in vitro roughly according to typical concentration-response curves obtained for the differentiation of ES cells and for the viability of ES and 3T3 cells in the presence of a concentration range of the test chemical.

Strongly embryotoxic chemicals usually inhibit differentiation of ES cells at very low concentrations and many of them also show a high cytotoxic potential at low concentrations both to 3T3 mouse fibroblast cells and ES cells, e.g. cytostatic drugs [5-fluorouracil (5-FU); see also fig. 1]. Chemicals characterized by a high teratogenic potential, e.g. retinoic acid (RA), are inhibiting differentiation at very low concentrations, while cytotoxic effects

are apparent at concentrations that are several orders of magnitude higher (fig. 2a). On the other hand, weakly embryotoxic chemicals are effective in the EST at intermediate concentrations, where the differentiation of ES cells into cardiomyocytes is more sensitive than growth inhibition, e.g. in case of the anticonvulsant drug diphenylhydantoin (DPH) (fig. 2b). Chemicals that do not exhibit any embryotoxic potential, e.g. the sweetener saccharine or the antibiotic penicillin G, are neither inhibiting growth nor differentiation of ES cells at concentrations of more than 500 µg/ml. Other nonembryotoxic chemicals, however, as for instance the vitamin and food additive ascorbic acid (ASC), exert cytotoxic effects at relatively low concentrations on differentiated 3T3 cells, whereas cell differentiation and/or viability of ES cells are less sensitive (fig. 2c).

Improved Prediction Model

A biostatistical evaluation was conducted on the limited set of 9 chemicals and the positive control 5-FU that were tested in our laboratory during prevalidation of the EST [Scholz et al., 1999]. In the process of validation, in vitro tests have to prove their reliability and relevance. For the first requirement, the reliability, individual labo-

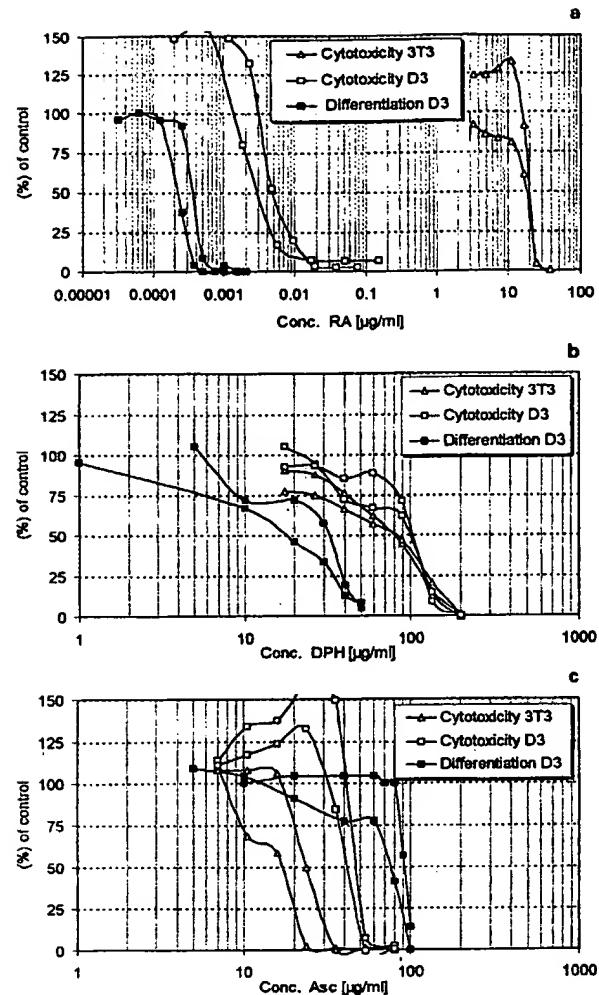


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Fig. 1. Endpoints and variables used in the improved PM. Diagram shows concentration-response curves for the three endpoints: cytotoxicity of D3 and 3T3 cells and inhibition of differentiation of D3 cells. The curves were obtained from two individual experiments using the positive control chemical 5-fluorouracil (5-FU). Endpoints ($IC_{50}D3$, $IC_{50}3T3$, ID_{50}) and the variable:

$$\left(\frac{IC_{50}3T3 - ID_{50}}{IC_{50}3T3} \right)$$

are marked by arrows.



2

Fig. 2. Typical concentration-response curves. **a** Concentration-response curves for the three endpoints of the EST: cytotoxicity of D3 and 3T3 cells and inhibition of differentiation of D3 cells. The curves were obtained from two individual experiments using the strongly embryotoxic chemical retinoic acid (RA). **b** Concentration-response curves of two individual experiments using the weakly embryotoxic diphenylhydantoin (DPH). **c** Concentration-response curves of two individual experiments using the nonembryotoxic ascorbic acid (Asc).

ratories verify a sufficiently high level of exactness by repeating results independently [Balls et al., 1990, 1995]. The second requirement, the relevance of an in vitro test for a specific application, is estimated by applying a biostatistically based prediction model (PM). For developing a scientifically acceptable in vitro embryotoxicity test, the PM should allow to discriminate between three classes of embryotoxicity; non-, weakly and strongly embryotoxic according to in vivo data.

The PM originally developed for the EST [Spielmann et al., 1997] is based on a mathematical calculation, the linear discriminant analysis [Norušis, 1994], taking into account three endpoints used to discriminate between the

three embryotoxicity classes: cytotoxicity of 3T3 fibroblasts and ES cells and differentiation of ES cells into contracting cardiomyocytes after 10 days of culture.

The concentration-response curves were analyzed in order to determine appropriate endpoints that allow to discriminate between the three classes of embryotoxic chemicals. First, 50% inhibition concentrations (IC_{50}) were calculated from the concentration-response curves for cytotoxicity of D3 cells and 3T3 fibroblasts and for differentiation of D3 cells, in order to evaluate whether individual endpoints alone sufficiently allow to discriminate between the three classes of embryotoxicity.

Table 2. Improved PM for the EST: linear discriminant functions I, II and III and classification criteria (class I–3)

Function I	$5.92 \lg(\text{IC}_{50}3\text{T3}) + 3.50 \lg(\text{IC}_{50}\text{D3}) - 5.31 \frac{\text{IC}_{50}3\text{T3} - \text{ID}_{50}}{\text{IC}_{50}3\text{T3}} - 15.7$
Function II	$3.65 \lg(\text{IC}_{50}3\text{T3}) + 2.39 \lg(\text{IC}_{50}\text{D3}) - 2.03 \frac{\text{IC}_{50}3\text{T3} - \text{ID}_{50}}{\text{IC}_{50}3\text{T3}} - 6.85$
Function III	$-0.125 \lg(\text{IC}_{50}3\text{T3}) - 1.92 \lg(\text{IC}_{50}\text{D3}) + 1.50 \frac{\text{IC}_{50}3\text{T3} - \text{ID}_{50}}{\text{IC}_{50}3\text{T3}} - 2.67$
Class 1: nonembryotoxic	If I > II and I > III
Class 2: weakly embryotoxic	If II > I and II > III
Class 3: strongly embryotoxic	If III > I and III > II

Second, figures 1 and 2 indicate that the variability of the IC_{50} is higher compared to the 25% inhibition concentrations (IC_{25}). Therefore, the discriminating potential of the two endpoints (IC_{25} , IC_{50}) had to be evaluated. Although the IC_{25} showed a better performance in the analysis of discriminance – and therefore provided a better discrimination – the IC_{50} was chosen as the appropriate endpoint for the following reasons. When testing chemicals under blind conditions, it is sometimes difficult to completely cover the entire range of the concentration-response curve between 100 and 0% both for viability and differentiation. Furthermore, given the intrinsic variability of the assay and the limited number of concentrations that can be tested within one experiment, the dilution factor should be sufficiently small to achieve the most exact measurement of the endpoint. Therefore, the concentration-response curves may not reach 25% viability or differentiation in each case, whereas the 50% inhibition concentrations are more easily met. Thus, the 50% viability and differentiation (IC_{50} , ID_{50}) was applied as endpoint in the linear discriminant analysis in the improved prediction model.

Yet the endpoint value (IC_{50} , ID_{50}) cannot be used directly as a variable in the linear analysis of discriminance. In order to convert the numerical value of the endpoint into a linear model the logarithm of the IC_{50} is calculated. The term 'endpoint' is used only for the 50% cytotoxic concentrations ($\text{IC}_{50}3\text{T3}$, $\text{IC}_{50}\text{D3}$) and the 50% differentiation concentration (ID_{50}), that are directly calculated from the concentration-response curve. The term 'variable' is applied to all mathematical calculations (logarithm, relative distance) of the original endpoints. The improvement of the PM described here is based on the same endpoints as the original PM, but uses a slightly different variable.

In order to include occasionally divergent test results on viability and differentiation into the model, the relative distance between two of the endpoints ($\text{IC}_{50}3\text{T3}$, $\text{IC}_{50}\text{D3}$) was used as a variable (fig. 1). This variable is independent from the absolute concentration values, since this information is already given by the two other variables, the $\lg(\text{IC}_{50}3\text{T3})$ and the $\lg(\text{IC}_{50}\text{D3})$ values.

The in vitro test provided a total of 9 different experimental variables [$\lg(\text{IC}_{50}3\text{T3})$; $\lg(\text{IC}_{50}\text{D3})$; $\lg(\text{ID}_{50})$; $\lg(\text{IC}_{25}3\text{T3})$; $\lg(\text{IC}_{25}\text{D3})$; $\lg(\text{ID}_{25})$; relative distance between $\text{IC}_{50}3\text{T3}$ and $\text{IC}_{50}\text{D3}$; relative distance between $\text{IC}_{50}3\text{T3}$ and ID_{50} , and relative distance between $\text{IC}_{50}\text{D3}$ and ID_{50}], each of which may contribute to distinguishing between the three groups of embryotoxic chemicals. Consequently, a stepwise selection of variables was performed [Bortz et al., 1993; Norušis, 1994; Backhaus et al., 1996]. This procedure identifies the best variable to discriminate between the selected classes. Subsequently, in a stepwise fashion, each of remaining variables is separately added to the model and rejected again, if it does not significantly improve the separation of three classes of embryotoxicity. Three variables were accepted in the analysis of discriminance which improved the distinction between the three embryotoxic classes compared to the original model. The linear discriminant functions of the PM for the EST incorporating the variables selected are shown in table 2.

The following procedure is ensued to classify the chemicals according to the improved PM: 50% inhibition concentrations ($\text{IC}_{50}3\text{T3}$, $\text{IC}_{50}\text{D3}$, ID_{50}) and the relative distance between $\text{IC}_{50}3\text{T3}$ and ID_{50} are determined and employed in the three linear discriminant functions. A chemical is classified as not embryotoxic, if the result of equation I exceeds the results of equations II and III. A chemical is classified as weakly embryotoxic, if the result of equation II exceeds the results of equations I and III. Finally, if the result of equation III exceeds the results of

Table 3. Classification of the test chemicals

Embryotoxicity in vivo	Number of chemicals	Number of cases	Prediction in vitro		
			1	2	3
Class 1: nonembryotoxic	3	9	9 (100.0)	0 (0)	0 (0)
Class 2: weakly embryotoxic	3	9	1 (11.1)	8 (88.9)	0 (0)
Class 3: strongly embryotoxic	4	12	0 (0)	1 (8.3)	11 (91.7)

The 10 chemicals were repeatedly tested, resulting in 30 individual experiments (number of cases). Correct classifications are italicized. 100% of the nonembryotoxic chemicals, 89% of the weakly embryotoxic chemicals and 92% of the strongly embryotoxic chemicals were classified correctly. In total 94% of all cases were classified correctly. To identify the endpoints, linear discriminant analysis was used [Bortz, 1993; SPSS, stepwise discriminant analysis, Norušis, 1994; Backhaus et al., 1996]. Figures in parentheses represent percentage.

equations I and II, the chemical is classified as strongly embryotoxic.

The classification of the so-called 'learning sample' according to the improved PM provided 94% correct classifications (table 3). However, a model usually fits the learning sample better than it will fit new chemicals. Thus the percentage of correctly classified cases is an overly optimistic estimation and a lower rate for correct classifications must be expected when new chemicals are evaluated with the model. Currently, a set of 20 chemicals is tested in a validation study of three in vitro embryotoxicity tests, including the EST. The results of this study will be used to evaluate the improved PM.

EST Detects Various Types of Teratogenic/Embryotoxic Chemicals

Teratogenic chemicals are distributed in numerous types of chemicals and function via diverse pathways, either highly specific receptor signalling pathways, or more general mechanisms. Although the molecular functions of several teratogens are the subject of intensive research, such as the RA receptor pathway or the arylhydrocarbon receptor pathway, the mechanisms of how adverse effects are affecting normal mammalian development are highly complex and have not sufficiently been identified. Therefore, establishing a highly specific, mechanistic test is impossible to date. Taking into account known mechanisms of embryotoxicity we have used the differentiation of ES cells into contracting cardiomyocytes as a means to measure differentiation and early mammalian development. Our results show that a variety of strong and weak teratogens can correctly be identified

in the EST, even chemicals which do not specifically act on early heart development in vivo. For instance, in vivo exposure of early postimplantation rodent embryos to *all-trans*-RA frequently results in craniofacial defects and defects of the central nervous system. Exposure at later developmental stages is often associated with limb and genitourinary defects [Kochhar, 1997, for effects of RA on cardiac development, see Rohwedel et al., 1999]. Nevertheless, RA used under our treatment conditions is a highly potent inhibitor of cardiomyocyte differentiation in vitro, and we did not obtain false-negative results in the EST so far during test development and prevalidation. However, it must be kept in mind that teratogens which specifically interfere with the development of other tissues, e.g. the palate or nervous system, may not be detected in the EST.

The EST and the improved PM for the EST are based on the different responsiveness of pluripotent ES cells and differentiated fibroblast cells to teratogenic/embryotoxic agents. This difference, especially with respect to the cytotoxicity tests with the two cell lines, may to some extent be influenced by the different media requirements and cell growth characteristics of D3 and 3T3 cells. On the one hand, media for 3T3 cells contain less serum (10% FCS) than media for the D3 cells (20% FCS). Since it is known that chemicals can have different affinities for serum proteins, the concentration of free and active compounds may be different in the presence of different serum concentrations, and therefore a higher sensitivity of 3T3 cells in the cytotoxicity test would be expected. On the other hand, 3T3 cells have a longer doubling time than D3 cells and display contact inhibition, which could also explain a higher sensitivity of D3 cells and cytotoxicity tests in some cases.

Since the EST is the only mammalian *in vitro* alternative to animal testing in developmental toxicology to date which is based on established cell lines and does not require pregnant animals, this test offers the possibility to be optimized for automated high throughput screening systems in toxicity testing of drugs and other chemicals for regulatory purposes. An earlier and slightly different approach to using ES cells in an *in vitro* embryotoxicity test, which used colony formation as a morphological endpoint of differentiation, showed a very poor prediction and had not been developed further [Newell and Beedles, 1994].

Further Strategies in the Improvement of the EST

Attempts to further optimize the EST test protocol are currently under way in order to identify endpoints of differentiation other than microscopic evaluation of contracting areas. This aim may be achieved by using promoter/reporter gene expression techniques and stably transfected ES cells which are expressing, for example, green fluorescent protein under the control of a developmentally regulated promoter, such as the cardiac α -actin promoter which is activated during cardiac muscle cell development [Kolossov et al., 1998]. Another approach may focus on endogenous gene expression by RT-PCR methods, for which protocols and primers exist to detect

early markers of gene expression in early development as well as gene expression patterns in developing EB with a very limited amount of ES cells [Wiles, 1993]. Such molecular techniques would offer the advantage of replacing the subjective evaluation through an experienced person by an objective measurement that can be automated, which is an important prerequisite for high throughput screening systems. Furthermore, the assay duration could be reduced, from 10 to e.g. 7 or even 5 days.

The inclusion of other endpoints in addition to cardiac muscle cell development is another important consideration in order to reduce the risk of obtaining false-negative results. For instance, early hematopoiesis and blood cell development can be induced in ES cell cultures under defined culture conditions and by the addition of specific growth factors.

Thus, there are several promising strategies to adapt or extend *in vitro* ES cell differentiation techniques in order to reduce animal testing in reproductive toxicology and at the same time to gain further insight into mechanisms of action of teratogens on the cellular and molecular level.

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